Distribution of Proteoglycans During the Hair Growth Cycle in Human Skin

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The involvement of proteoglycans in hair growth has been recognized through the observation of increased hair growth in diseases such as the mucopolysaccharidoses and pre-tibial myxedema, which involve an increase in skin proteoglycan content. In an attempt to understand this, we have examined the distribution of chondroitin 6 sulphate (C6S), unsulphated chondroitin (COS), dermatan sulphate (DS), and heparan sulphate proteoglycans (HSPG) in frozen tissue sections of normal scalp by immunostaining. Results show that during anagen, the thick connective tissue sheath around the follicle stains strongly for C6S, COS, and DS. COS is uniquely associated with this region and is not found beneath the epidermis or infundibular epithelium. HSPG is, however, localized in the basement membrane zone adjacent to the outer root sheath. In addition, all of these proteoglycans are localized in the dermal papilla. In mid-catagen, we observed significant loss of C6S and COS staining from both the dermal papilla and the connective tissue sheath, but no decrease in staining for HSPG. In late catagen, very little staining of C6S and COS was observed.

In early anagen, we observed that C6S was again present in the connective tissue sheath and dermal papilla; however, COS staining appeared to be weaker and less closely associated with the follicle. HSPG staining was observed in early anagen in a pattern very similar to that found for other basement membrane components. Results for DS were not obtained for catagen or early anagen. These results provide further evidence that hair growth is associated with the presence of chondroitin proteoglycans in the follicle environment and that the cessation of growth is associated with their removal. Further studies are underway to characterize the relationship between hair growth and proteoglycans. *J Invest Dermatol 96:191–195, 1991*

We have previously demonstrated that the distribution of basement membrane zone components such as fibronectin, laminin, type IV collagen, and bullous pemphigoid antigen is not uniform throughout the hair growth cycle in both rat and human skin [1–3]. This may indicate a role for these molecules in the dramatic changes in the growth and differentiation of the hair follicle during the hair growth cycle.

More recently, another group of matrix molecules, the proteoglycans, has emerged as having important functions as regulators of the growth and differentiation of many cell types, including epithelial and fibroblastic cells from skin [4,5].

Proteoglycans consist of a protein core to which a number of glycosaminoglycan side chains are attached. The characteristic feature of glycosaminoglycans is the repeating sequence of hexosamine and uronic acid residues, the different types being distinguished from each other by the nature of these sugars and their degree of sulphation. Proteoglycans usually carry only one type of glycosaminoglycan chain and are named after this component [6,7].

Although proteoglycans are an important component of the extracellular matrix, little is known of their role in regulating cellular behavior in skin and hair.

The observation of hypertrichosis in various disease states such as pretibial myxedema and the mucopolysaccharidoses [8–10], where there is excessive accumulation of proteoglycans or their glycosaminoglycan chains, suggests a possible role of proteoglycans in hair growth control. This speculative role is supported by previous histochemical studies that drew attention to the presence of glycosaminoglycan in the dermal papilla of the hair follicle and showed that staining was most intense during the growth stage [11].

As a further investigation of the role of proteoglycan in normal human hair growth, we have used specific antibodies to probe the distribution of proteoglycans in the follicle environment and changes in proteoglycan distribution during the different stages of the hair growth cycle.

MATERIALS AND METHODS

Preparation of Skin Sections Human skin samples were obtained during surgical removal of small scalp lesions and were snap-frozen in thawing isopentane without prior mounting. More than 40 samples of human scalp or beard skin were examined for localization of proteoglycans. The photomicrographs in this report were taken from seven such samples, including male and female scalp and male beard, from subjects varying from 30–60 years of age. Five- to eight-micrometer sections were cut onto microscopic slides pre-

Manuscript received January 23, 1990; accepted for publication June 28, 1990.

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Abbreviations:
BMZ: basement membrane zone
BSA: bovine serum albumin
C4S: chondroitin 4 sulphate
C6S: chondroitin 6 sulphate
COS: unsulphated chondroitin
DEJ: dermo-epidermal junction
DS: dermatan sulphate
HSPG: heparan sulphate proteoglycan
IAP: immunoalkaline phosphatase
IPx: immunoperoxidase
TBS: tris-buffered saline

0022-202X/91/$03.50

coated with 1 mg/ml poly-L-lysine (Sigma, St. Louis, MO), allowed to dry at room temperature for at least 1 h, and then stored at −20°C until use.

Antibodies Murine monoclonal antibodies recognizing chondroitin 6-sulphate proteoglycan (C6S), unsulphated chondroitin proteoglycan (COS), dermatan sulphate proteoglycan (DS), and chondroitin 4 sulphate proteoglycan (C4S) were purchased from ICN Biomedicals, High Wycombe, U.K. The original antigens were pre-digested with chondroitinase ABC, which digests the chondroitin side chains down to a disaccharide stub containing an unsaturated bond [12]. It is the configuration of this disaccharide and its sulphation position that confers the antigenicity of the molecule and finally determines the enzyme and antibody combination required to yield staining of the appropriate chondroitin proteoglycan. The antigenic site is re-exposed in the tissue by prior treatment of the section with either chondroitinase AC (C4S and C6S) or ABC (C6S, COS, and DS) (ICN Biomedicals) [12,13]. The specificity and characterization of these antibodies has been determined previously [12]. A polyclonal rabbit antiserum to heparan sulphate proteoglycan (anti-HSPG) was used [14].

To distinguish between C4S and DS, as both proteoglycans are recognized by the same antibody [12], it is necessary to compare sections stained after treatment with chondroitinase AC (C4S only) and chondroitinase ABC (DS and C4S).

Immunostaining Procedures Standard indirect immunoperoxidase (IPx) and immunoalkaline phosphatase (IAP) staining techniques have been used to localize proteoglycans in frozen tissue sections by light microscopy; IPx has been used for immunoelectronmicroscopy.

The majority of our studies were done using IPx staining. However, some later staining was carried out by IAP as part of investigations into improved procedures for localization.

For IPx, sections were brought to room temperature, air-dried for 60 min, fixed in either cold ethanol or acetone for 10 min, and again air-dried. The area on the slide around the sections was marked with a water-repellant solution containing 15% dimethyl polysiloxane, so that the area covered by the antibody would be limited. Sections were incubated with chondroitinase ABC or AC (Seikagaku Kogyo Co. Ltd., Tokyo) at 0.2 U/ml in tris acetate buffer, 0.01 M, pH 7.0, for 30 min. Controls received no enzyme treatment.

The sections were washed and then incubated for 20 min in 5% normal swine or rabbit serum diluted in tris-buffered saline (TBS), 0.05 M, pH 7.6, containing 1% bovine serum albumin (BSA) (Sigma), as a blocker of non-specific binding of the peroxidase-conjugated antibody. The blocking serum was drained off and primary antibody was applied. The monoclonal antibodies were diluted 1:200, and anti-HSPG, 1:100 in TBS/BSA. Sections were incubated for 60 min at room temperature, washed 3 times for 5 min in TBS/BSA, and the enzyme-conjugated antibody was applied.

Rabbit anti-mouse IgG, conjugated with horseradish peroxidase and diluted 1:100 in TBS/BSA (Dako, High Wycombe, UK), was used for identifying binding of monoclonal antibodies.

A peroxidase-conjugated swine anti-rabbit IgG antibody (Dako) was used to mark binding of the anti-HSPG antibodies. In some cases, a triple layer staining procedure was adopted: peroxidase-conjugated swine anti-rabbit IgG (1:100) was applied after the rabbit anti-mouse conjugate. In these cases, swine serum was used for blocking. All conjugate incubations were for 30 min and were followed by three 5-min washes. All sections were incubated with diaminobenzidine substrate 0.5 mg/ml containing 0.01% H_2O_2 for 10 min, after which the reaction was stopped by adding excess TBS. The sections were finally rinsed, counterstained in hematoxylin (Shandon, Runcorn, UK) for 30 sec, dehydrated through alcohol into xylene, and mounted in DPX.

For IAP, the staining procedure was identical to that for IPx staining except an alkaline phosphatase-conjugated second antibody (Dako) was used at a 1:20 dilution, and Fast Red/Napthol AS-MX phosphate (Sigma) was used as substrate. The substrate solution was prepared by dissolving 10 mg Fast Red and 2 mg AS-MX phosphate in 1 ml each of tris buffer (0.1 M, pH 8.2). These were filtered sequentially through Whatman No 1. paper, followed by 7 ml buffer. Finally, 2.5 mg levamisole, dissolved in 1 ml distilled water, was filtered into the final solution. Preparation in this way was essential to prevent precipitation.

The substrate solution was applied for 15–20 min and the reaction stopped by flooding the sections with TBS. Levamisole is used to block endogenous alkaline phosphatase activity.

Immunoelectronmicroscopy Skin sections at 8–10 μm were cut onto melinex strips and air-dried. The melinex had been precoated with poly-L-lysine. The staining was carried out as described for IPx staining at light microscope level.

Sections were post-fixed in osmium tetroxide (1%) in 0.1 M Na cacodylate buffer, pH 7.4, for 30 min, rinsed in water for no less than 30 min, and dehydrated through a graded alcohol series. The sections were finally soaked in epoxy resin (TAAB) and set at 60°C for 48 h. The melinex was snapped off and 100-nm sections were mounted onto copper grids and viewed with a Jeol JEM-100C electron microscope.

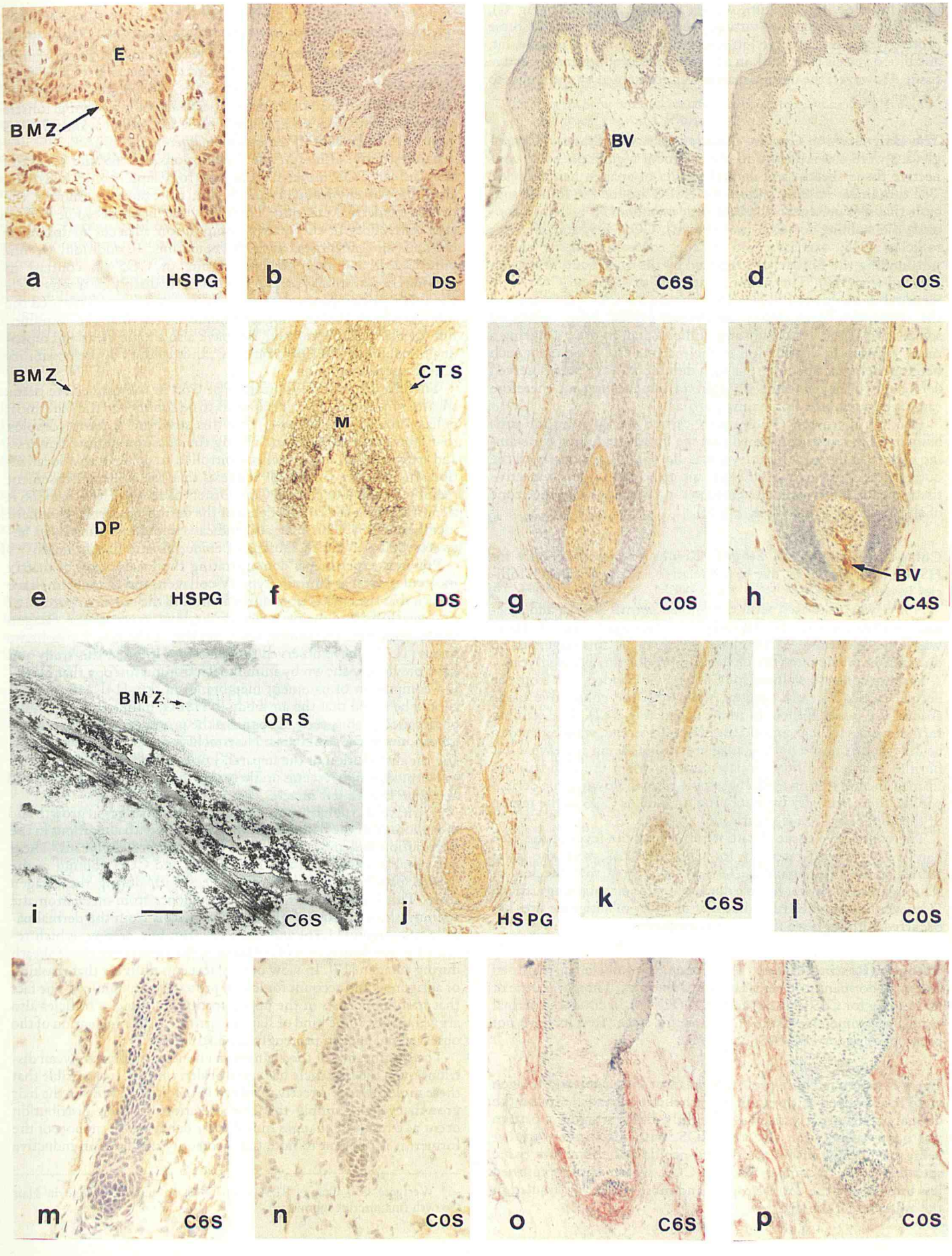
Photography Photographs were taken on Fuji XD100 and XD400 color print film.

Results The staining of proteoglycans in human scalp skin revealed a distinct heterogeneity in the distribution of the different types of proteoglycans studied. In particular, there was a striking non-uniform staining pattern of chondroitin proteoglycan throughout the hair growth cycle.

Distribution of Proteoglycan in Skin The staining of HSPG was observed in the basement membrane zone (BMZ) underlying the epidermis and associated with all other basement membrane-bearing structures in the skin (Fig 1*a,e*). DS staining was intense in all areas of connective tissue in the dermis, including the dermal papilla matrix (Fig 1*b,f*), and C4S was present predominately in blood vessels, with little or no staining of the dermal matrix itself (Fig 1*h*).

The follicular staining patterns for C6S and COS were similar to each other. However, the results of staining with the antibody to COS are novel in that previous characterization of this antibody did not demonstrate the presence of COS in skin, but only in cartilage and aorta [12,13]. A major difference in the distribution of these proteoglycans was observed at the dermal epidermal junction and

Figure 1. Light and electron microscope localization of proteoglycans in human scalp by IPx *(a–n)* and IAP *(o, p)* staining techniques. *a–d* show the general staining pattern seen at the dermo-epidermal junction *(DEJ)* with antibodies to HSPG *(a)*, DS *(b)*, C6S *(c)*, and COS *(d)*. Note that there is no staining of COS at the DEJ, in contrast to HSPG and C6S. *e–h* show the distribution of HSPG *(e)*, DS *(f)*, COS *(g)*, and C4S *(h)* in association with follicles in anagen. The staining for C6S is not shown but was identical to that seen here for COS. The localization of C6S at the ultrastructural level is shown in *i*. Note the distribution of staining throughout the outer layers of orthoganol collagen and in the loose connective tissue. There is also surface staining on cells within the connective tissue sheath. Note there is no staining in the region of the BMZ adjacent to the follicle outer root sheath epithelium. *j–l* show the distribution of proteoglycans associated with a follicle in mid-catagen. Note there has been loss of most bulbar epithelium and that in comparison with HSPG *j*, the staining of C6S *(k)* and COS *(l)* is greatly reduced in this region; *m* and *n* show telogen follicles stained with C6S and COS, respectively. Note that the dermal papilla and connective tissue associated with the lower follicle is not stained. The distribution of C6S and COS associated with an early anagen follicle is shown in *o* and *p*, respectively. Note the epithelial growth below the club hair and concomitant re-expression of C6S and COS within the connective tissue sheath and dermal papilla. *a*, *m*, and *n*, magnification × 200; *b–l*, *o*, and *p*, magnification × 100. *Line bar* on *i*, 1 μm. E, epidermis; BV, blood vessel; CTS, connective tissue sheath; DP, dermal papilla; M, melanin (black granular material); BMZ, basement membrane zone; ORS, outer root sheath.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upper follicle infundibulum, where, in contrast to C6S (Fig 1*c*), COS (Fig 1*d*) staining was virtually absent. The expression of these proteoglycans was observed in association with nerve, muscle, fat, and blood vessels (Fig 1*c,d*), in addition to the follicular connective tissue. However, the dermal matrix was not stained by antibodies to C6S or COS.

The Hair Growth Cycle—Anagen (Growth Stage) Proteoglycans were observed within the basement membrane zone, connective tissue sheath, and dermal papilla of anagen hair follicles. The thin linear staining pattern for HSPG around the follicle suggests that it is present in the basement membrane (Fig 1*e*). In contrast, the staining for C6S (not shown), COS, DS, and, in some follicles, C4S, was observed in the connective tissue sheath surrounding the follicle, as suggested by the wide band of fibrillar staining (Fig 1*f,g*). The staining patterns for C6S and COS were identical in the lower follicle. The staining for C4S was variable in the connective tissue sheath and dermal papilla, being associated solely with blood vessels in some follicles and, in others, having a more widespread distribution in the matrix. The reasons for such heterogeneity are not clear, but such differences have been observed between two anagen follicles within the same section and therefore cannot be attributed to a staining artifact.

Electron microscopy studies were carried out by using the antibody to C6S; results confirmed that the fibrillar nature of the staining pattern observed at light level was due to an association with the outer layers of orthogonal collagen and cells in the outer connective tissue sheath. C6S was not detected in the basement membrane zone of the lower anagen follicle (Fig 1*i*).

Catagen (Transitional Stage) Results are described only for HSPG, C6S, and COS, due to the relative scarcity of catagen follicles in scalp skin.

HSPG was associated with the follicle dermal papilla and basement membrane zone during early to mid-catagen (Fig 1*j*). There was some thickening of the glassy membrane at this stage, but no diminution in the intensity of the staining for this proteoglycan.

In sharp contrast, staining for C6S and COS faded, first from the dermal papilla in early catagen, then from the connective tissue around the lower follicle in mid-catagen (Fig 1*k,l*). The staining further up the follicle, around the epithelial strand, was observed to be different from the normal anagen staining, being somewhat less fibrillar and more diffuse. In late catagen, when the club hair has formed and only a thin epithelial strand extends down to the dermal papilla, there was very little staining for C6S or COS in the region of the connective tissue sheath (not shown).

The connective tissue sheath at and above the level of the club hair always stained intensely for C6S and COS, with the latter fading at the infundibulum. This demonstrated that the loss of staining from the lower follicle does not represent a staining artifact. However, it cannot be ruled out that masking of antigenic sites has occurred, although we think this is unlikely.

Telogen (Resting Phase) At telogen, the follicle has regressed to a small dormant structure, carrying a club hair. There was little or no staining for C6S (Fig 1*m*) and COS (Fig 1*n*), in either the dermal papilla or connective tissue sheath at this stage. Results have not been obtained for DS, C4S, or HSPG.

Early Anagen Those proteoglycans lost in catagen and telogen were re-expressed as the follicle epithelium expanded around the dermal papilla in early anagen. A slightly different staining pattern was observed between C6S and COS, with the former staining being specifically within the dermal papilla and connective tissue sheath (Fig 1*o*), in comparison with COS, which was re-expressed less intensely in the dermal papilla and more widely distributed in the adjacent dermis (Fig 1*p*).

DISCUSSION

The results described here reveal the presence of at least four different types of proteoglycan associated with normal-growing human hair follicles. The dermal papilla matrix is especially rich in these molecules, containing C6S, COS, HSPG, DS, and probably C4S. The connective tissue sheath that surrounds the growing follicle contains C6S, COS, and probably some DS, whereas only HSPG appears to be present in the basement membrane zone.

Interestingly, although the distributions of C6S and COS are very similar, COS, unlike C6S, appears to be largely restricted to the perifollicular environment and is not, for example, found at the dermal-epidermal junction. There may be, therefore, some important differences in the matrix composition adjacent to follicular epithelia in comparison with the epidermis. In addition, we obtained clear and reproducible staining of COS, in contrast to previous observations [12,13]. It is not immediately clear why there should be a discrepancy, however. In our studies, we have used the more sensitive IPx staining technique rather than the immunofluoresence used previously. We have also shown clear and reproducible staining of COS in rat skin*, thus ruling out the possibility of interspecies variation.

The ultrastructural localization of C6S in the connective tissue sheath is intriguing in that it appears to be confined to the outermost collagenous layers, showing that this structure is more complex than previously thought and raising the question of how synthesis and extracellular deposition is controlled to achieve this localization. In addition, this result suggests that the follicular basement membrane does not contain C6S. Others have suggested that C6S is a constituent of the lamina densa of the dermo-epidermal basement membrane [15]. This may also indicate important differences between the basement membrane of epidermis and hair follicles.

Previous experiments demonstrating the localization of matrix molecules, such as laminin, type IV collagen, and fibronectin, have shown that the basement membrane around the transient portion of the hair follicle remains intact throughout most of the catagen stage, with some thickening being evident as the glassy membrane forms [1]. We have observed the same for HSPG in this study and have previously shown by immunoelectronmicroscopy that HSPG is a component of basement membranes in skin [14]. However, it should be noted that the antibody to HSPG, unlike the other antibodies used in this study, recognizes the protein core of the proteoglycan, not the GAG chains. No conclusions can be drawn regarding the distribution of the heparan sulphate side chains during hair growth, although it seems unlikely that they would be degraded and the protein core left intact.

We have also demonstrated that the cyclic changes in growth and morphology of the hair follicle are associated with alterations in the distribution and apparent amount of certain proteoglycans. These are, notably, disruption and loss of C6S and COS staining in the dermal papilla and connective tissue sheath during the catagen stage. This is consistent with earlier evidence from metachromatic staining showing loss of glycosaminoglycans from the dermal papilla in catagen [11,16] and from electron microscopy, which revealed active destruction of collagen in the connective tissue sheath during catagen [17]. In view of this, it seems unlikely that masking of antigens could account for loss of proteoglycan staining. The fact that staining persists in the upper portions of catagen follicles also argues against some kind of staining artifact as an explanation of the observed changes in proteoglycan distribution.

The significance of the changes in chondroitin proteoglycan distribution in the hair cycle has yet to be determined. It is possible that these molecules are directly involved in mediating events in the hair growth cycle or simply that the alterations in their distribution occur as a result of changes in follicular behavior. In support of the former, it is relevant to note that proteoglycans have an inductive

* Westgate GE, Craggs RI, Gibson WT: Immune Privilege in Hair Growth (manuscript submitted).

effect on hair growth, both when injected into the skin [18] and in diseases that result in an accumulation of mucopolysaccharides in the dermis [8–10).

During anagen, the cells of the bulb matrix do not express markers of class I MHC [19,20]. We have recently suggested, on the basis of parallel rat skin studies, that the chondroitin proteoglycans may exert a protective functional barrier around the follicle in anagen, thus preventing recognition of the matrix cells by mediators of natural cytotoxicity [20]. Examples of such a protective role for proteoglycans have been observed previously, most notably at the fetal-maternal interface [21,22]. By implication, this suggests that perhaps catagen involves immune-mediated destruction of the epithelium. In support of this, we have demonstrated that during catagen a population of activated macrophages becomes progressively more closely associated with the regressing follicle. This event occurs at the time of the loss of chondroitin proteoglycan in the dermal papilla and connective tissue sheath [23]. These cells may contribute to the loss of proteoglycan through enzymatic degradation and also to the cytotoxic removal of the follicular epithelium. Further studies are underway to confirm the presence of such cells in human catagen.

We have not been able to study the distribution of DS or C4S throughout the hair growth cycle. It is likely the DS is involved in the stabilization of collagen during fibrillogenesis in the dermis and in dermal elements such as the connective tissue sheath and dermal papilla [24].

In conclusion, we have clearly demonstrated that the distribution of certain proteoglycans is heterogeneous throughout the hair growth cycle and we suggest that in anagen these proteoglycans may confer on the follicle a status of immune privilege and, as such, provide a protective environment for maintenance and growth.

We gratefully acknowledge Dr. R. I. Craggs for helpful discussions, Mr. A. C. Weaver for electron microscopy, Miss M. A. Blount for contribution to the immunostaining, Miss E. A. Cooper for help with preparation of the manuscript, and Mr. D. Chance for the photographic reproduction.

REFERENCES

1. Couchman JR, Gibson WT: Expression of basement membrane components through morphological changes in the hair growth cycle. Devel Biol 108:290–298, 1985
2. Westgate GE, Shaw DA, Harrap GJ, Couchman JR: Immunohistochemical localization of basement membrane components during hair follicle morphogenesis. J Invest Dermatol 82:259–264, 1984
3. Westgate GE, Weaver AC, Couchman JR: Bullous pemphigoid antigen localization suggests an intracellular association with hemidesmosomes. J Invest Dermatol 84:218–224, 1985
4. Bernfield M, Banerjee SD: The turnover of basal lamina glycosaminoglycan correlates with epithelial morphogenesis. Devel Biol 90:219–305, 1982
5. Turley EA: Proteoglycans and cell adhesion. Cancer Metastasis Rev 3:325–399, 1984
6. Hay ED: Extracellular matrix. J Cell Biol 91:205s–223s, 1981
7. Silbert JE: Structure and metabolism of proteoglycans and glycosaminoglycans. J Invest Dermatol 79:31s–37s, 1982
8. McKusick VA: The Mucopolysaccharidoses — Inheritable Disorders of Connective Tissue. The CV Mosby Co., St Louis, 1966, pp 352–399
9. Muenzer J: Mucopolysaccharidoses. Adv Pediatr 33:269–302, 1986
10. Diseases of the hair and scalp, Chapter 8. In: Rook A, Dawber R (eds.). Blackwell Scientific Publications, London, Boston, Melbourne, 1982, pp 248–252
11. Montagna W, Chase HB, Malone HJD, Melarango HP: Cyclic changes in polysaccharides of the papilla of the hair growth. J Micros Sci 93:241–245, 1952
12. Caterson B, Christner JE, Baker JR, Couchman JR: Production and characterisation of monoclonal antibodies directed against connective tissue proteoglycans. Fed Proc 44:386–393, 1985
13. Couchman JR, Caterson B, Christner JE, Baker JR: Mapping by monoclonal antibody detection of glycosaminoglycans in connective tissue. Nature 307:650–652, 1984
14. Davis MAF, Gibson WT, Minter HJ, Smith CG, Couchman JR: Characterisation of an antiserum to a basement membrane heparan sulphate proteoglycan. Biochem Soc Trans 15:1076–1077, 1987
15. Fine JD, Couchman JR: Chondroitin 6 sulphate containing proteoglycan: a new component of human skin dermoepidermal junction. J Invest Dermatol 90:283–288, 1988
16. Sylven B: Distribution of metachromatic material during hair growth. Exp Cell Res 1:582–589, 1950
17. Parakkal PF: Role of macrophages in collagen resorption during the hair growth cycle. J Ultrastr Res 29:210–217, 1969
18. Mayer K, Kaplan D, Steigleder GK: Effect of acid mucopolysaccharides on hair growth in the rabbit. Proc Soc Exp Biol Med 108:59–63, 1961
19. Harrist TJ, Ruiter DJ, Mihm MC, Bhan AK: Distribution of major histocompatibility antigens in human skin. Br J Dermatol 109:623–633, 1983
20. Westgate GE, Craggs RI, Gibson WT: Do proteoglycans confer immune privilege on growing hair follicles? (abstr) J Invest Dermatol 92:A541, 1989
21. Bradbury S, Billington WD, Kirby DRS, Williams EA: Surface mucin of human trophoblast. Am J Obst and Gynec 104:416–418, 1969
22. Currie GA, Bagshawe KD: The masking of antigens on trophoblast and cancer cells. The Lancet I:708–710, 1967
23. Craggs RI, Westgate GE, Gibson WT: Distribution of immune cells in rat skin during the normal hair growth cycle (abstr). J Invest Dermatol 92:A415, 1989
24. Lindahl V, Hook M: Glycosaminoglycans and their binding to biological macromolecules. Annu Rev Biochem 47:385–417, 1978